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Purification and Molecular Characterization of the Novel Highly Potent Bacteriocin TSU4 Produced by *Lactobacillus animalis* TSU4

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Abstract Bacterial infections causing fish diseases and spoilage during fish food processing and storage are major concerns in aquaculture. Use of bacteriocins has recently been considered as an effective strategy for prevention of bacterial infections. A novel bacteriocin produced by *Catla catla* gut isolates, *Lactobacillus animalis* TSU4, designated as bacteriocin TSU4 was purified to homogeneity by a three-step protocol. The molecular mass of bacteriocin TSU4 was 4117 Da determined by Q-TOF LC/MS analysis. Its isoelectric point was ~9. Secondary conformation obtained by circular dichroism spectroscopy showed molecular conformation with significant proportions of the structure in α-helix (23.7 %) and β-sheets (17.1 %). N-terminal sequencing was carried out by the Edman degradation method; partial sequence identified was NH_2-SMSGFSKPHD. Bacteriocin TSU4 exhibited a wide range of antimicrobial activity, pH and thermal stability. It showed a bacteriocidal mode of action against the indicator strain *Aeromonas hydrophila* MTCC 646. Bacteriocin TSU4 is the first reported bacteriocin produced by fish isolate *Lactobacillus animalis*. The characterization of bacteriocin TSU4 suggested that it is a novel bacteriocin with potential value against infections of bacteria such as *A. hydrophila* MTCC 646 and *Pseudomonas aeruginosa* MTCC 1688 and application to prevent spoilage during food preservation.

Keywords Bacteriocin · Fish disease · *Lactobacillus animalis* · Antimicrobial activity · Food preservation · Aquaculture

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Introduction

Bacteriocins are ribosomally synthesized extracellularly released bioactive antimicrobial peptides/proteins (AMPs) produced by numerous bacteria that kill or inhibit the growth of other bacteria and show great potential against antibiotic-resistant strains [1, 2]. Bacteriocins produced by Gram-positive bacteria are often small, heat-stable, hydrophobic and membrane-permeabilizing cationic peptides with fewer than 60 amino acid residues [2]; they possess broad spectrum inhibitory activity through bacteriocidal and bacteriostatic mode of action. Bioactivity, stability, toxicity and biochemical properties are important performance parameters of bacteriocins to be considered during application in food industries [3].

Bacteriocins produced by lactic acid bacteria (LAB) have been shown to improve the aquatic environment in both shrimp and fish aquaculture [4]. LAB are considered as an important component of aquaculture production due to their ability to inhibit growth of pathogenic and spoilage-inducing bacteria [5–7]. LAB exert strong antagonistic activity against many related and unrelated microorganisms, including pathogenic and food spoilage bacteria such as *Aeromonas*, *Pseudomonas*, *Listeria*, *Clostridium*, *Bacillus* and *Staphylococcus* spp. [8, 9]. The inhibitory activity of LAB is attributed mainly to their production of antimicrobial compounds such as hydrogen peroxide, diacetyl and AMPs. Among these, AMPs are the major antimicrobial compounds that disrupt the integrity of bacterial cell membranes. They have been explored as an alternative to antibiotics to control pathogenic bacteria for prevention of bacterial infections and spoilage during food preservation [10–12].

The most frequent bacterial pathogen of fishes is *Aeromonas hydrophila* (recently named *Aeromonas sobria*), which affects a wide variety of freshwater fish species [9]. It can easily spread through accidental abrasions thereby causing exophthalmia, skin infections, gastroenteritis, haemorrhagic septicaemia, abscesses, ulcers, abdominal distension, small superficial lesions and local haemorrhages in the gills and opercula [13]. It is also a ubiquitous bacterium that can cause a broad spectrum of human diseases, including septicaemia, meningitis, wound infections and gastroenteritis [14].

In our previous study, we identified and characterized *Lactobacillus animalis* TSU4 a potent broad spectrum probiotic strain which inhibited bacterial fish pathogens such as *A. hydrophila*, *Pseudomonas aeruginosa* and other food spoil pathogens [15]. At the same time, we established that the cause of the inhibition was the antimicrobial activity of a protein molecule. This study describes the purification and molecular characterization of the bioactive antimicrobial protein, which is designated as bacteriocin TSU4. Its application would range from preventing the bacterial infections and spoilage during food preservation as well as against human gastrointestinal bacterial pathogens.

Materials and Methods

Bacterial Strains and Growth Conditions

For the production of bacteriocin TSU4, fish isolate, *L. animalis* TSU4 (NCBI accession no. KJ412485) was grown in deMan Rogosa Sharpe (MRS) broth at 37 °C for 24–48 h under microaerophilic conditions [15]. *A. hydrophila* MTCC 646, routinely used as an indicator

strain, was grown in Luria-Bertani (LB) broth under similar conditions. Specific growth of strain TSU4 was measured spectrophotometrically at 600 nm (model 2467; Shimadzu, Kyoto, Japan). All media components were purchased from Hi-Media (Mumbai, India). Other chemicals of analytical grade were procured from Sigma-Aldrich (USA).

Bacteriocin Activity Assay

The isolate, *L. animalis* TSU4, was inoculated in MRS medium to an initial OD_{600} of 0.02. After overnight growth, cell-free supernatant was obtained by centrifugation (10,000*g*, 15 min at 4 °C), filtered through a 0.2-μm membrane filtered (Millipore, USA) and checked for bacteriocinogenic activity in terms of arbitrary unit AU/ml. Briefly, 150 μl LB, 50 μl of bacteriocin at 2-fold serial dilutions and 50 μl of indicator organism (OD_{600}, 0.02) were mixed in separate wells in a microtitre plate. These plates were incubated for 4 h at 37 °C, and the growth inhibition of the indictor organism was measured spectrophotometrically at 600 nm using a microtitre plate reader (Bio-Rad, USA) and compared with an untreated sample. One AU was defined as the amount of bacteriocin that inhibited the growth of the indicator organism by 50 % when compared with the untreated control [16].

Protein Quantification

The amount of protein in samples was determined by the method of Bradford (1976) [17] using a calibration curve developed with bovine serum albumin as protein standard.

Purification of Bacteriocin TSU4

The purification of bacteriocin TSU4 was performed using a three-step protocol as described earlier with slight modifications [18] by using advance chromatography resin. In brief, the protocol involved positive salt precipitation (step 1) by ammonium sulphate to enrich bacteriocin in the form of precipitate and remove contaminating media components in the supernatant. Further purification was done by cation exchange chromatography (CEC; step 2) followed by reverse phase chromatography (RPC; step 3) to assess the level of purification.

Step 1: Positive Salt Precipitation

The purification was conducted using a 1000-ml culture of strain TSU4 grown to the early stationary phase. The cells were discarded by centrifugation (10,000*g*, 15 min, 4 °C), and the bacteriocin present in the supernatant was filtered through a 0.2-μm filter (Millipore, USA) to remove any residual cells and then subjected to precipitation by ammonium sulphate 60 % (*w*/*v*) with stirring at 4 °C for 6 h. The precipitate was collected by centrifugation (10,000*g*, 10 min, 4 °C). The precipitate was dissolved in 60 ml of sodium phosphate buffer (20 mM, pH 5.0; buffer A). The sample was desalted by dialysing (2.0 kDa cut-off membrane, Sigma, USA) against the same buffer. The dialysed suspension was centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant was filtered through a 0.2-μm membrane and checked for bacteriocin activity in terms of AU/ml using *A. hydrophila* MTCC 646. The same filtered supernatant was labelled as precipitation output (P OP) and then further purified by CEC.

Step 2: Cation Exchange Chromatography

The P OP obtained from salt precipitation was applied to a cation exchange resin Fractogel EMD SO_3^- (M) (Merck Millipore, Germany), packed volume 17 ml in a Vantage Laboratory Column, VL 11×250 (Millipore, USA) on an AKTA purifier system (GE Healthcare, Uppsala, Sweden). The column was equilibrated with buffer A at a flow rate of 4.5 ml/min, and bacteriocin was eluted with a NaCl gradient (0–100 % of 1 M) in sodium phosphate buffer (20 mM, pH 6.0; buffer B) in 10-ml fractions at the same flow rate. The eluted protein was monitored at 280 nm, and conductivity was measured in millisiemens per centimetre. Eluted protein peak fractions were tested for bacteriocin activity and determined in AU/ml. The fractions exhibiting activity were pooled and labelled as cation exchange chromatography output (CEC OP) and then further purified by RPC.

Step 3: Reverse Phase Chromatography

The CEC OP was applied to a 3-ml pre-pack column, Resource RPC (GE Healthcare, Uppsala, Sweden) connected to an AKTA purifier system. The column was equilibrated with sodium phosphate buffer (20 mM, pH 6.0; buffer C) at a flow rate of 1 ml/min, and bacteriocin was eluted with a linear gradient of 0–100 % buffer C containing 40 % 2-propanol (Merck, Germany). The flow rate was maintained at 1.0 ml/min throughout the run. Eluted protein was monitored by a UV detector at 280 nm, and peaks were fractioned. The active bacteriocin containing part of peak was pooled, designed as bacteriocin TSU4, lyophilized and stored at −20 °C for further study.

Purity Confirmation by Reverse Phase High-Performance Liquid Chromatography

The purity of bacteriocin TSU4 was confirmed by injecting a 20-μl sample into an analytical HPLC system (Agilent 1200 series HPLC system, Germany) equipped with an analytical column (ACE-3, C-18-300, 100×2.1 mm; ACE capillary column). The solvents used were solvent A (0.1 % TFA in Milli-Q water) and solvent B (0.1 % TFA in acetonitrile). A 38-min linear gradient from 0 to 100 % solvent B at a flow rate of 0.3 ml/min was used throughout the run; the eluted peak was monitored at 210 nm. The purity confirmation of P OP sample was carried out using same method.

Molecular Size Determination

Tricine Sodium Dodecyl Sulphate Poly Acrylamide Gel Eletrophoresis Analysis

To determine the molecular mass of bacteriocin TSU4, the active pooled fraction eluted after RPC was subjected to tricine sodium dodecyl sulphate poly acrylamide gel eletrophoresis (SDS-PAGE) [19] with some modifications, which included a 10–20 % linear gradient layer of 49.5 % T and 6 % C separating gel. A natural polypeptides SDS-PAGE standard (1.5–26.6 kDa) protein marker from Bio-Rad was used to compare protein sizes. An optimized run condition for ~3 h was used (constant current 15 mA, max voltage up to 300 V during stacking and constant current 30 mA during separating). The gel was cut into two vertical parts after the run. The part of the gel containing the sample and the protein marker was stained with silver nitrate, while the remaining part, containing only the sample, was washed in 10 mM

phosphate buffer (pH 7.0) for 4 h and then fixed and subjected to direct detection of antimicrobial activity by overlaying with LB soft agar (~15 ml) containing 50 μl of a 5-h culture of indicator strain, *A. hydrophila* MTCC 646.

Mass Spectrometry Analysis

Mass spectrometry analysis of bacteriocin TSU4 was carried out using an AB SCIEX TripleTOF 4600 LC/MS System (AB SCIEX, USA) as described by Dimitrijevic et al. [20] with some modification. In brief, the mass spectrometer was run in positive electron spray ionization (ESI) mode with mass/charge (m/z) ratio in the range of 100–3200 m/z and a quadrupole–time of flight (Q-TOF) mass analyser was used. Ten microlitres of sample containing bacteriocin TSU4 (0.4 mg/ml) was loaded to analytical HPLC column Pursuit 3 Diphenyl 150×2.0 mm (Agilent Technologies) equipped with an ACQUITY Ultra Performance Liquid Chromatography (UPLC) system (Waters Corporation, USA); the mobile phase was solvent A (0.1 % TFA in Milli-Q water) and solvent B (0.1 % TFA in acetonitrile) at a flow rate of 0.2 ml/min. The data were analysed by Analyst TF 1.6 software.

Isoelectric Point Determination

The isoelectric point (pI) value was determined using the method described earlier [21] with some modifications. Purified bacteriocin TSU4 was subjected to isoelectric focusing (IEF) in a Phast electrophoresis system (GE Healthcare, Sweden) using Phast dry IEF precast gel (GE Healthcare, Sweden) composed of 5 % acrylamide gel that contained an ampholyte with a pH range of 3 to 10. Optimized run conditions were used at coolant temperature 10 °C: pre-focusing condition 2000 V, 3-mA current for 75 AVh (~10 min); sample load condition 200 V, 3-mA current, up to 90 AVh (~3 min); and pI value separation condition 2000 V, 3 mA current, up to 410 AVh (~20 min). After the run, the gel was incubated for 1 h in a fixing solution of 10 % (*v*/*v*) trichloroacetic acid in Milli-Q water. The protein bands were visualized by silver nitrate staining.

N-terminal Amino Acid Sequencing

The purified bacteriocin TSU4 was subjected to Edman degradation and analysed on a protein/peptide sequencer, model 494 Procise (Perkin Elmer Applied Biosystems, USA) with an online amino acid analyser (model 140C, PTH Perkin Elmer Applied Biosystems, USA). Partial sequence of bacteriocin TSU4 was carried out.

Circular Dichroism and Fluorescence Emission Spectrum

Circular dichroism (CD) spectra were recorded from 260 to 200 nm at 25 °C using a JASCO model J815 spectrometer (JASCO analytical instruments, Japan) purged with N_2 gas [22]. Stock solutions of bacteriocin TSU4 were prepared in Milli-Q water, and CD spectra were recorded for bacteriocin TSU4 solutions at a final concentration of 50 μg/ml. Instrument parameters were the following: response time 1 s, scan speed 50 nm/min, sensitivity 50 m/degree, step resolution 0.1 nm and bandwidth 5 nm. CD spectrum of the matrix buffer was subtracted from the CD spectra of the bacteriocin TSU4 solution to eliminate the interference from cell, optical equipment and matrix buffer. High-frequency noise was reduced by means of

low-path Fourier-transform filtering. The ellipticity was expressed as the mean-residue molar ellipticity in degrees per square centimetre dmol. The secondary structure composition was estimated by deconvolution, as implemented by Spectra manager software (version 2.03.0, Build 1). The fluorescence emission spectrum was recorded from 290 to 540 nm using a JASCO model J815 spectrometer at 25 °C [22]. Instrument parameters were mode emission, ex wave length 280 nm, sensitivity 700 V, DIT 4, and ex band width 3.00. The data were evaluated by Spectra manager software (version 2.03.0, Build 1).

Antimicrobial Spectrum

The antimicrobial spectrum of bacteriocin TSU4 was evaluated by using a well diffusion assay [23]. The activity of the purified sample was determined by a critical dilution assay and adjusted to 400 AU/ml. This solution was applied to a 6-mm well on the surface of a BHI/LB agar plate overlaid with molten soft BHI/LB agar (BHI/LB broth supplemented with 0.8 % agar (*w*/*v*) inoculated with 1 % (*v*/*v*) of 24-h culture of the indicator strains such as *A. hydrophila* MTCC 646, *P. aeruginosa* MTCC 1688, *Shigella flexneri* MTCC 1457, *Salmonella typhimurium* MTCC 733, *Staphylococcus aureus* MTCC 737, *Salmonella paratyphi* MTCC 735, uropathogenic *Escherichia coli* (UPEC) MTCC 729 and *E. coli* MTCC 443. The plates were then incubated overnight at 37 °C, and the diameters of the inhibition halos were measured (mm) after incubation.

Effects of Different Treatments on Bacteriocin TSU4 Activity

The nature of the antimicrobial activity of bacteriocin TSU4 was investigated by treating with non-proteolytic enzymes such as catalase (pH 7.0) and α-amylase (pH 7.0) at 1 mg/ml concentration and proteolytic enzymes such as pepsin (pH 2.0), trypsin (pH 7.0) and proteinase K (pH 7.0) (Sigma-Aldrich, USA) at three different concentration (0.1, 1 and 5 mg/ml). All reactions were performed at 37 °C for 2 h, followed by deactivation by heating in boiling water for 5 min. The residual activity was determined using the well diffusion method. The specific buffers for each enzyme were used as controls. Thermal stability of bacteriocin TSU4 was tested by incubating bacteriocin aliquots at 40 °C for 2 h and 60, 80 and 100 °C for 30 min. The effects of different pH on bacteriocin activity were tested by adjusting the pH of bacteriocin aliquots from pH 2.0 to 10.0 at increments of 2 pH units using 1 M NaOH or HCl. After incubation at 37 °C for 2 h, the pH of each sample was readjusted to 6.0. The inhibition activities of all treated and control samples were determined and expressed as percentage of residual antimicrobial activity with respect to the control which was set at 100 % [24].

Mode of Action

The mode of action of bacteriocin TSU4 was tested against the indicator strain, *A. hydrophila* MTCC 646. Bacteriocin TSU4 with 200 AU/ml was applied to actively growing cells of indicator strain at the early exponential phase (~10^6 colony-forming units (CFU)/ml). The indicator microorganism growing in LB medium in the absence of bacteriocin TSU4 was used as a control. Changes in the turbidity of the cultures were recorded by measurement of the OD at 600 nm, and the number of CFU was determined by plating the samples on LB agar media.

Results

Purification of Bacteriocin TSU4

In order to ascertain the presence of a bacteriocin and to study its properties, the cell-free supernatant was subjected to sequential purification steps. The increase in the specific activity and purification obtained at each step is summarized in Table 1. After ammonium sulphate precipitation, an almost 2-fold increase in specific activity was observed (Table 1). In CEC on Fractogel SO_3^-, one major absorbance peak along with a small peak was eluted between 20 and 50 % of 1.0 M NaCl. Fractions number 11–15 (Fig. 1a) eluted at ~30 mS/cm conductivity showed antimicrobial activity. When the same fractions were pooled together, purity of bacteriocin showed an almost 4-fold increase in purification. Two separate peaks were observed in the Resource RPC column (Fig. 1b); the tailing portion of the second peak fractions showed specific activity of 180.55 AU/μg and resulted in 6.4-fold increase in purification. The purity profiles of P OP sample and purified bacteriocin TSU4 from reverse phase high-performance liquid chromatography (RP-HPLC) analysis with retention time of 15.6 min are shown in Fig. 1c, d, respectively. However, the final yield was only ~2 %.

Molecular Mass and Antimicrobial Activity

The molecular mass of purified bacteriocin TSU4 analysed by tricine SDS-PAGE showed a band at ~4 kDa (Fig. 2a). This molecular mass of bacteriocin was confirmed by Q-TOF LC/MS analysis which gave a molecular mass of 4117 Da (Fig. 3). The determination of antibacterial activity against *A. hydrophila* MTCC 646 showed a growth inhibitory zone at the same position as visualized in the stained gel (Fig. 2a).

Isoelectric Point

The net charge and pI of purified bacteriocin TSU4 were determined by isoelectric focusing electrophoresis. Bacteriocin TSU4 co-migrated towards the cathode, giving a pI of ~9 (Fig. 2b).

Table 1 Purification of bacteriocin TSU4 produced by the *Lactobacillus animalis* TSU4 strain

Purification step	Volume (mL)	Total protein (μg)	Total activity (AU)[a]	Specific activity (AU/μg)[b]	Purification fold[c]	Yield (%)[d]
Cell free supernatant (CFS)	1000	89,086	2,511,768	28.19	1.00	100.00
Positive salt precipitation	60	17,047	991,546	58.17	2.06	39.48
Cation exchange chromatography	50	1668	216,762	129.95	4.61	8.63
Reverse phase chromatography	20	305	55,068	180.55	6.40	2.19

[a] Determined by critical dilution assay, considering the total sample volume and expressed in arbitrary units (AU)

[b] Determined by the ratio between the total bacteriocin activity and the total protein content, expressed in AU/μg

[c] Determined by the ratio between the specific activity of individual step and cell free supernatant step

[d] Determined by the ratio between the total activity of individual step and cell-free supernatant step

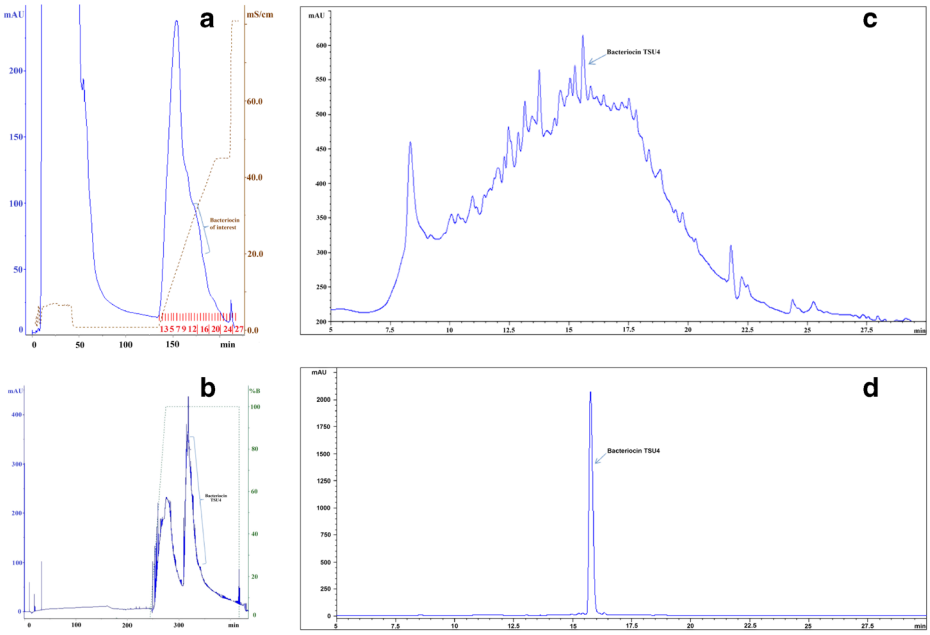

Fig. 1 **a** Chromatogram of cation exchange chromatography of bacteriocin TSU4 purification (UV 280 nm in mAU is shown by a *continuous line* and conductivity in millisiemens per centimetre is shown by a *dash line*), pooled fraction 11 to fraction 15 shown activity. **b** Chromatogram of reverse phase chromatography of bacteriocin TSU4 purification (UV 280 nm in mAU is shown by a *continuous line* and percentage of buffer B is shown by a *dash line*), tailing fraction of second peak contains purified bacteriocin TSU4. **c** RP-HPLC chromatogram of P OP sample after salt precipitation (retention time 15.6 min). **d** RP-HPLC chromatogram of purified bacteriocin TSU4 (retention time 15.6 min)

N-terminal Amino Acid Sequencing

N-terminal sequencing was provided for the first ten amino acid residues: NH_2-Ser-Met-Ser-Gly-Phe-Ser-Lys-Pro-His-Asp. The partial sequence was compared with the known entries in the NCBI database on the basis of protein–protein homology, BLASTP (http://blast.ncbi.nlm.nih.gov/). The comparison showed that the sequenced residues were not identical to any known bacteriocins/proteins. This led us to infer that bacteriocin TSU4 is a unique and novel peptide.

Molecular Conformation

The secondary structural conformation of bacteriocin TSU4 was investigated using CD spectroscopy. As shown in Fig. 4a, bacteriocin TSU4 adopts a defined and rigid structure. The CD spectrum showed minima at 206 and 222 nm, and the ellipticity at 222 nm versus the ellipticity at 206 nm (*R* value) was 2.1. It showed that significant parts of bacteriocin TSU4 were in α-helical and in β-sheet conformations (23.7±0.2 %) and (17.1±0.1 %), respectively, and remaining parts were random coil (59.2±1 %). The fluorescence emission maximum at 280-nm excitation of bacteriocin TSU4 was 356±1 nm (Fig. 4b).

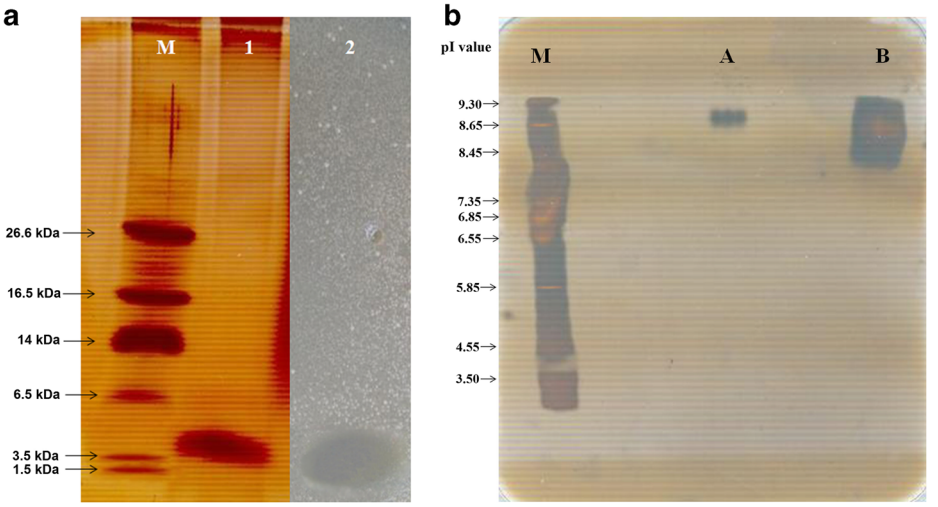

Fig. 2 **a** Tricine-SDS-PAGE of bacteriocin TSU4 stained by silver nitrate: Unstained marker (*lane M*), purified bacteriocin TSU4 (*lane 1*) and portion of the SDS-PAGE gel overlaid with LB soft agar containing *A. hydrophila* MTCC 646 strain (*lane 2*). **b** Isoelectric focusing electrophoresis gel for pI value determination of bacteriocin TSU4: pI value maker (*lane M*), bacteriocin TSU4 load volume 1 μl (*lane A*) and bacteriocin TSU4 load volume 4 μl (*lane B*)

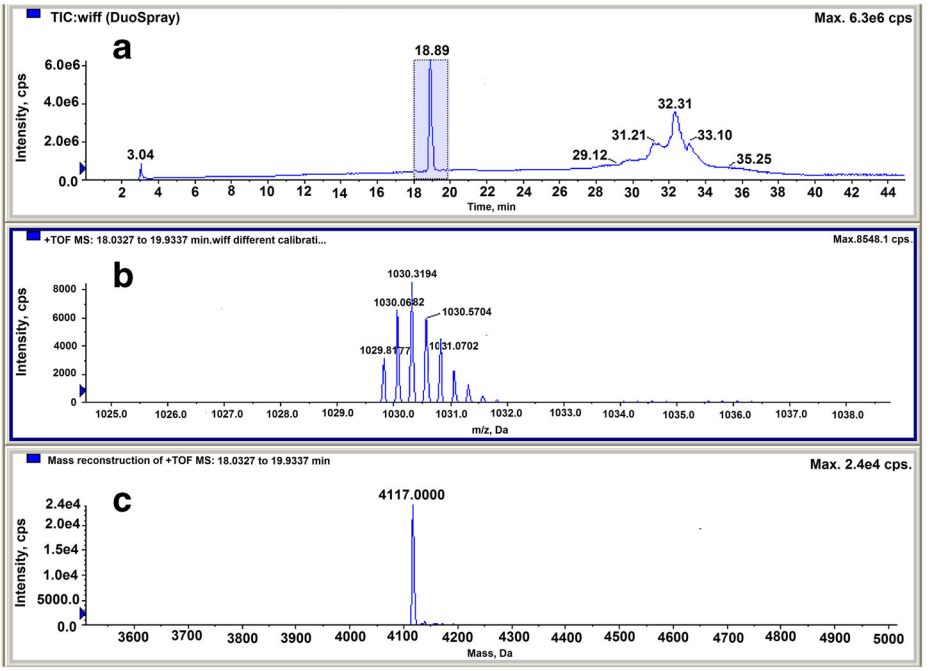

Fig. 3 Molecular mass analysis by Q-TOF LC/MS. **a** Total ion chromatogram (TIC) of bacteriocin TSU4. **b** Mass/charge ratio (m/z) of bacteriocin TSU4. **c** Mass of bacteriocin TSU4 in Da

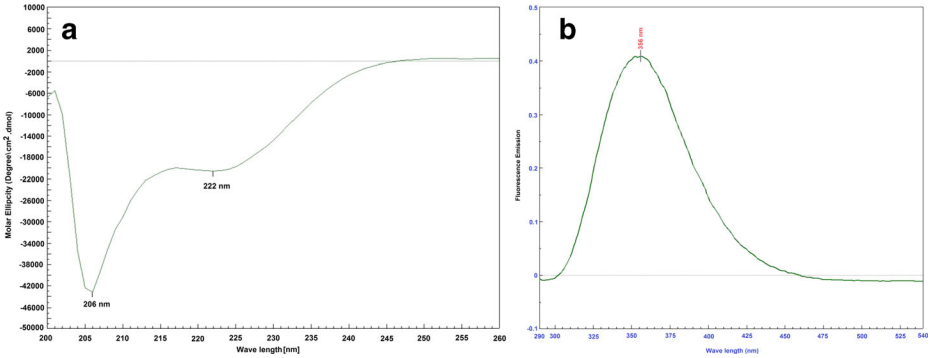

Fig. 4 **a** CD spectrum of bacteriocin TSU4. CD spectra were recorded from 260 to 200 nm. The ellipticity was expressed as the mean-residue molar ellipticity in degrees per square metre dmol. **b** Fluorescence emission spectrum of bacteriocin TSU4 upon excitation at 280 nm

Antimicrobial Spectrum

Bacteriocin TSU4 inhibited all tested pathogens; the inhibition zone ranged from 23 to 32 mm (Table 2). The strongest inhibition zone (32 mm) was against *Shigella flexneri* MTCC 1457, and the weakest inhibition zone (23 mm) was against *Salmonella paratyphi* MTCC 735.

Effect of Different Treatments in Bacteriocin TSU4 Activity

With increasing concentration to 1 mg/ml of proteolytic enzymes, trypsin and pepsin antimicrobial activity was reduced to 34.26±2.24 and 40.63±2.24 %, respectively. Activity completely vanished at 5 mg/ml of trypsin or pepsin, or at 0.1 mg/ml of proteinase K (Table 3). In addition, the bacteriocin activity was stable at a wide pH range between 2 and 10. High thermal stability was also observed for bacteriocin TSU4; residual activity was 62.33 ±2.1 % after treatment at 100 °C for 30 min (Table 3).

Table 2 Antimicrobial spectrum of bacteriocin TSU4 against various target strains

Target strain	Collection	Growth medium	Growth temperature (°C)	Inhibition (mm)
Aeromonas hydrophila	MTCC 646	LB	37	28±1
Escherichia coli	MTCC 443	LB	37	27±1
Uropathogenic *E. coli* (UPEC)	MTCC 729	LB	37	28±2
Pseudomonas aeruginosa	MTCC 1688	BHI	37	25±1
Salmonella typhimurium	MTCC 733	LB	37	26±1
Salmonella paratyphi	MTCC 735	LB	37	23±1
Shigella flexneri	MTCC 1457	BHI	37	32±2
Staphylococcus aureus	MTCC 737	BHI	37	25±2

LB Luria-Bertani agar, *BHI* brain heart infusion agar (Himedia)

Table 3 Different treatments on inhibition activities of bacteriocin TSU4

Treatment		Residual antimicrobial activity (%)
Enzyme	Concentrations (mg/ml)	
Catalase	1	83.50±1.50
α-Amylase	1	91.13±0.95
Trypsin	0.1, 1 and 5	61.42±1.66, 34.26±2.24 and 0
Proteinase K	0.1, 1 and 5	0, 0 and 0
Pepsin	0.1, 1 and 5	69.30±1.97, 40.63±2.24 and 0
pH		
2	2 h	68.52±1.24
4	2 h	81.11±0.50
6	2 h	94.35±2.51
8	2 h	71.00±2.00
10	2 h	64.42±1.78
Temperatures (°C)		
40	2 h	100
60	30 min	91.42±1.56
80	30 min	80.56±0.80
100	30 min	62.33±2.1

Values are presented as the mean±SEM (n=3)

Mode of Action

Bacteriocin TSU4 cause a drop in log CFU/ml of viable cells of *A. hydrophila* MTCC 646 from 6.0 to 3.4 over a period of 11 h. In the control sample (without bacteriocin TSU4), viable cells of *A. hydrophila* MTCC 646 reached ~10^{10} CFU/ml after 11 h of growth. The OD of this indicator microorganism remained constant after the addition of the bacteriocin TSU4 (Fig. 5). These results indicate that bacteriocin TSU4 exhibited a bacteriocidal effect against the pathogenic bacterium *A. hydrophila* MTCC 646.

Discussion

Although there are various remedies available for fish diseases, use of bacteriocin for prevention of fish diseases is an advanced alternative [13]. Currently, use of LAB and other Gram-positive bacteria are attractive for the production of natural bioactive potent molecules with potential therapeutic applications. One such compound is the peptide biomolecule called as antimicrobial peptide. Several lactobacilli produce bacteriocins (antimicrobial peptides) that are known to have potential applications for inhibiting the pathogenic bacteria causing fish diseases [25] and as active agents in food preservation [8]. The production of bacteriocin by such strains may have additional benefits such as medicinal properties. *L. animalis* C060203 isolated from soil produced bacteriocin that exhibited antibacterial activity against *L. sakei* JCM 1157 [26].

There are many approaches used for protein purification; of these, hybrid multi-step purification processes are most prominent. Hybrid approaches are the optimized strategy that

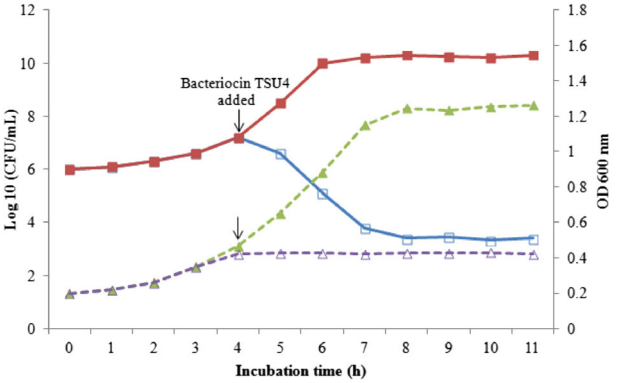

Fig. 5 The effect of bacteriocin TSU4 on the growth of *A. hydrophila* MTCC 646. OD at 600 nm in the absence (*black triangle*) and presence (*white triangle*) of bacteriocin TSU4. Viable cell counts (CFU/ml) in the absence (*black square*) and presence (*white square*) of bacteriocin TSU4. The *arrow* indicates the point at which the bacteriocin TSU4 was added

allows the separation of bacteriocin from other process- and product-related impurities. Bacteriocin TSU4 shares many properties with other bacteriocins purified using the same strategy in other studies [18, 27, 28]. Bacteriocin TSU4 is cationic and hydrophobic, as demonstrated by CEC and RPC, in agreement with earlier reports on bacteriocins [18, 28]. In this study, we achieved homogeneous purification of bacteriocin TSU4 as demonstrated by RPC. The reliability of each purification step was demonstrated by a significant increase in the specific activity of bacteriocin TSU4, as reported earlier for other bacteriocins [18].

Tricine SDS-PAGE may not reveal the accurate molecular mass of bacteriocin TSU4, so the exact mass spectrum was also measured using Q-TOF LC/MS analysis. Its results identified a molecule with low molecular mass <10 kDa with hydrophobic and cationic properties, expected to have one membrane-spanning segment that could be involved in its mode of action, since a membrane pore-forming bacteriocin indicates membership of class II bacteriocins.

It is important to purify a bacteriocin prior to its characterization. RP-HPLC is one of the most common and robust analytical techniques that have been used for checking purity of many bacteriocins [24, 29]. In this study, the purity of bacteriocin TSU4 was determined by the RP-HPLC; the single peak observed indicates absolute purity of the sample. Bacteriocins have been reported to have isoelectric point (pI) ranging from 8.6 to 10.4 [30]. Their net positive charge varies with pH which is important for bacteriocin efficacy and purification. Bacteriocin TSU4 had an alkaline pI value indicating its strong cationic property under a wide range of pH.

Comparing the N-terminal amino acid sequence of bacteriocin TSU4 with other bacteriocins using protein–protein BLAST in NCBI showed no apparent homology with other known bacteriocins. This apparent lack of similarities suggests that bacteriocin TSU4 is a novel AMP. As per the N-end rule described by Gonda et al. [31], determination of the half-life period of the protein mostly depends on the N-terminus amino acid residue. The N-terminal amino acid of the bacteriocin TSU4 is serine. Approximate half-life of proteins in mammalian systems with serine in N-terminal residues is >1.9 h [31]. Plantaricin Y showed strong inhibitory activity against *Listeria monocytogenes* BCRC 14845 (a fish pathogen) that contains serine as the N-terminal amino acid, which accords with our findings [32].

CD spectroscopy is a valuable tool, especially for showing changes in secondary molecular conformation. It simultaneously reveals thermodynamic information and verifies native structure. Although quantitative interpretation of CD spectra of bacteriocins with high tryptophan content is difficult [22], it seems reasonable to conclude that significant parts of bacteriocin TSU4 are in α-helix and β-sheet conformations which clearly distinguish it from other bacteriocins, which tend to be flexible and unstructured in aqueous solution [33]. The conformation of bacteriocin TSU4 may be responsible for its biological activity. The fluorescence emission maximum at 356 nm is characteristic of tryptophan residues in a polar environment [22] and indicates the presence of tryptophan in bacteriocin TSU4.

An interesting feature of bacteriocin TSU4 was that it had activity against several Gram-negative pathogens, a property which is uncommon for most known, if not all, bacteriocins from Gram-positive bacteria; this feature also suggests that bacteriocin TSU4 is a novel AMP.

Important features of bacteriocin TSU4, from the viewpoint of its possible application in food products, are its thermostability and sensitivity to proteolytic enzymes [24, 34]. The antimicrobial activity of the bacteriocin TSU4 was partly or completely inactivated by proteolytic enzyme treatments at different final concentrations. This revealed that the active moiety of the antimicrobial substance was proteinaceous. Additionally, no changes in activity were observed after treatments with non-proteolytic enzymes catalase and α-amylase, implying that hydrogen peroxide and carbohydrate were not responsible for inhibition.

The general mode of action of bacteriocin has been reported to be bacteriocidal due to its pore-forming action on the cell membrane making the cell leaky [35]. Bacteriocin TSU4 also had bacteriocidal action against *A. hydrophila*. In comparison to untreated cultures, the killing of cells by bacteriocin TSU4 was highly effective and quick. A bacteriocidal mode of action has previously been reported for several bacteriocins: bacteriocin PJ4 produced by *Lactobacillus helveticus* PJ4 [24] and caseicin TN-2 by *Lactobacillus casei* TN-2 [36].

Conclusion

Our study demonstrates the potential of bacteriocin TSU4 as a natural potent therapeutic biomolecule that could act against common freshwater fish pathogens such as *A. hydrophila* and *P. aeruginosa* for prevention of fish diseases as well as other food spoilage pathogens during food preservation. Biochemical studies revealed that bacteriocin TSU4 was partially heat-stable, even after heating at 100 °C for 30 min, and active over a wide pH range (2–10). The result from Q-TOF LC/MS analyses showed that the active peptide (bacteriocin TSU4) had a precise molecular mass of 4117 Da, and N-terminal sequencing data revealed that similar bacteriocin is not still discovered. Taken together, the results suggest that bacteriocin TSU4 is a novel and promising candidate for future application as an antimicrobial agent that could prevent fish diseases caused by bacterial infections and inhibit spoilage during food preservation. Further in vivo evaluation and safety profile studies are required to determine its immunogenicity, toxicity and importance in real-life applications.

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